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Diagnosis of Human Congenital Cytomegalovirus Infection by Amplification of Viral DNA from Dried Blood Spots on Perinatal Cards

Lori Scanga,* Shu Chaing,† Cynthia Powell,‡§ Arthur S. Aylsworth,‡§ Lizzie J. Harrell,¶‖ Nancy G. Henshaw,‖ Chris J. Civalier,* Leigh B. Thorne,* Karen Weck,* Jessica Booker,* and Margaret L. Gulley*

From the Department of Pathology and Laboratory Medicine, Pediatrics,‡ and Genetics,§ The University of North Carolina at Chapel Hill, Chapel Hill; The North Carolina State Laboratory of Public Health,† Raleigh; and the Department of Molecular Genetics and Microbiology¶ and the Clinical Microbiology Laboratory,‖ Duke University Medical Center, Durham, North Carolina*

Congenital human cytomegalovirus (HCMV) infection affects 1% of children and is the most common infectious cause of sensorineural hearing loss. Due to the difficulty of diagnosing deafness and other neurological disorders in infants, affected individuals may not be recognized until much later when active infection has resolved and culture is no longer informative. To overcome this problem, congenital HCMV infection was diagnosed retrospectively by testing residual blood samples collected from newborns and dried on perinatal cards as part of the North Carolina Newborn Screening Program. We modified the Qiagen method for purifying DNA from dried blood spots to increase the sample size and recovery of the lysate. A multiplex, real-time TaqMan polymerase chain reaction assay on an ABI 7900 instrument measured a highly conserved segment of the HCMV *polymerase* gene and the *APOB* human control gene. HCMV DNA was detected in blood dried on perinatal cards from all seven infants with culture-proven congenital infection, and all 24 negative control cases lacked detectable HCMV DNA. Our findings suggest that it is possible to diagnose congenital HCMV infection using dried blood collected up to 20 months earlier. Further studies are warranted on patients with hearing loss or other neurological deficits to determine the percentage that is attributable to congenital HCMV infection. ***(J Mol Diagn 2006, 8:240–245; DOI: 10.2353/jmoldx.2006.050075)***

Human cytomegalovirus (HCMV) infection is common in the general population and, when primary infection occurs early in pregnancy, can affect the developing neurological system leading to sensorineural hearing loss and other neurological sequelae. Congenital HCMV infection is defined as HCMV infection acquired up to 3 weeks of age, either *in utero* or perinatally via body fluids such as cervical mucous, breast milk, urine, or saliva.[1] Congenital HCMV infection is a significant cause of fetal and neonatal mortality and morbidity. It occurs in ~1% of all births and is symptomatic in 10 to 20% of affected infants.[2] Although symptomatic infection may also occur on reactivation of latent maternal infection, the majority of clinical sequelae and symptomatic infection is attributable to primary maternal HCMV infection. Symptomatic infection often involves multiple organs and is manifest as jaundice, hepatosplenomegaly, petechial rash, microcephaly, motor disability, chorioretinitis, cerebral calcifications, lethargy, respiratory distress, and seizures. Almost all symptomatic neonates have neurological sequelae, most commonly sensorineural hearing loss, and often developmental delay ranging from subtle learning disabilities to severe mental retardation. The majority of congenitally infected infants (80 to 90%) have no clinical signs or symptoms at birth, but 5 to 15% of these asymptomatic infected infants will later be found to have neurological sequelae, most commonly sensorineural hearing loss.[3]

When a newborn is suspected of being infected by HCMV, culture of the fetal urine or serological testing of the mother can help determine whether HCMV infection is the cause.[2] However, when congenital infection is not considered until later in childhood and after the active infection has cleared, it is then difficult or impossible to document congenital HCMV infection. Recent advances in DNA technology have helped to overcome this barrier to diagnosis after the newborn period. Retrospective diagnosis of congenital HCMV infection has been achieved by detecting HCMV DNA in dried blood samples applied to perinatal cards (Guthrie cards). Conventional end-

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Address reprint requests to Margaret L. Gulley, M.D., Dept. of Pathology, Brinkhous-Bullitt Building, University of North Carolina, Chapel Hill, NC 27599-7525. E-mail: margaret_gulley@med.unc.edu.

Table 1. Clinical Information on Seven Patients with Culture-Proven Congenital CMV Infection

Patient ID*	Age at positive urine culture (days)	Clinical findings
A	21	Deceased, pneumonia, hepatitis, twin of patient B
B	22	Deceased, pneumonia, hepatitis, twin of patient A
C	2	Anemia, jaundice, respiratory distress
D	4	Not known
E	1	Not known
F	11	Not known
G	5	Not known

* Perinatal cards were obtained anonymously from the State Health Laboratory, and thus experimental laboratory results could not be linked to clinical status. Patient B was included in the study even though the urine culture was not obtained until 1 day after the 21-day limit for defining congenital CMV infection because the patient's twin (patient A) was diagnosed within the limit, and both twins were symptomatic before 21 days.

point polymerase chain reaction (PCR) has been used to amplify HCMV DNA from perinatal cards of children with sensorineural hearing loss[4,5] and malformations of cortical development.[6,7] Newborn blood is routinely collected and dried on perinatal cards as part of state newborn screening programs. When residual blood cards are retained and made available for clinical use, it provides new avenues for HCMV detection.

In the current study, we validated a real-time amplification method for retrospective diagnosis of congenital HCMV infection. DNA was extracted from dried blood on perinatal cards and was subjected to real-time PCR amplification to determine the extent to which HCMV DNA was detectable in children known to have congenital HCMV infection. We modified the Qiagen method for extracting DNA from dried blood spots to increase the sample size and recovery of the lysate. Instead of using conventional end-point PCR, we chose real-time technology to minimize technologist time, to improve turn-around time, and to minimize the risk of amplicon contamination, all of which should streamline its implementation in clinical laboratories.

Materials and Methods

Patient Samples

Seven patients with culture-proven congenital HCMV infection were identified by searching clinical microbiology databases at University of North Carolina Hospitals and Duke University Medical Center throughout the previous 2 years (Table 1). HCMV infection was detected in each of the seven infants by urine culture at 1, 2, 4, 5, 11, 21, or 22 days after birth. At University of North Carolina Hospitals, urine for culture was collected and adjusted if necessary to a slightly pink color with 5% sodium bicarbonate. One ml of urine was added to 1.5 ml of ice-cold viral transport medium (Hanks' balance salt solution with 0.5% gelatin and antibiotics), and then 0.2 ml of this mixture (University of North Carolina Hospitals) or of undiluted urine (Duke University Medical Center) was inoculated into MRC-5 shell vials (human diploid fibroblasts; Diagnostic Hybrids, Inc., Athens, OH, or Viromed Laboratories, Minnetonka, MN), incubated for up to 72 hours, and subsequently stained with anti-HCMV monoclonal antibody (Light Diagnostics, distributed by Chemicon International, Temecula, CA). At University of North Carolina Hospitals, additional urine shell vial cultures were examined for viral cytopathic effect twice per week for 2 weeks and once per week until at least 21 days of culture. Alternatively, at Duke University Medical Center, conventional cell culture tubes (Diagnostic Hybrids, Inc.) were also inoculated with 0.2 ml of each patient specimen, centrifuged at 700 $\times$ *g* for 10 minutes, and incubated at 36°C for 14 days. Cultures were observed every other day for characteristic cytopathic effects of HCMV. After cytopathic effects developed, cells were scraped from the tube and HCMV was confirmed using fluorescent antibody targeting CMV immediate early antigens IE1 and IE2 as described above.

The perinatal cards from the seven infants with culture-proven congenital HCMV (positive controls) and negative control infants whose cards were filed in front of and behind the cards from the positive control patients were obtained from the North Carolina State Laboratory of Public Health. Perinatal blood cards are obtained within 48 hours of birth on all infants born in North Carolina for purposes of metabolic screening. Abnormal screening tests are followed up by a second blood card collection. In five of the seven positive control patients (patients A, B, D, E, and G), another card was collected at 6 to 33 days of age. Also, in patient F, two perinatal cards were collected within 48 hours of birth (at 24 hours and 33 hours). Because the cards from patients A and B were filed back-to-back for the first collection, the number of negative control cards for the first collection was 12, and the number of negative control cards for the second collection was also 12, for a total of 24 negative control cards. Each card generally contains five blood spots, each 1 cm in diameter. The cards were stored in the North Carolina State Laboratory of Public Health at room temperature in an air-conditioned environment for 2 to 20 months before DNA extraction for this study. This investigation was conducted under the supervision of our Institutional Review Board.

In addition to the aforementioned positive and negative control cases, we prepared mock samples from fresh blood obtained from our clinical archives. The viral load of each fresh blood sample had been previously measured using the same real-time PCR assay described below except that DNA was extracted from fresh whole blood using DNA Isolation Kit 1 on a Roche MagnaPure instrument (Roche Molecular Systems, Indianapolis, IN). The HCMV-negative patient blood had undetectable

HCMV by real-time PCR to a sensitivity of 400 copies per ml of blood and was also HCMV-seronegative (IgG and IgM). The HCMV-positive blood was from an AIDS patient with active HCMV infection as shown by high HCMV viral load measurement and signs of HCMV infection by physical examination. The blood was spotted onto perinatal cards (Schleicher and Schuell Inc., Keene, NH) in 50-μl aliquots and dried for at least 48 hours. The mock samples were used to assess the efficacy of extraction and amplification from dried blood spots. Finally, assay sensitivity and linearity was tested by spiking Towne strain virus into HCMV-negative blood at serial 10-fold concentrations. The spiked blood was then spotted onto perinatal cards, dried, extracted, and amplified to evaluate the performance characteristics of viral load measurement from perinatal cards.

Perinatal Card Preparation

The newborn blood spot specimens were collected according to NCCLS guidelines.[8] Briefly, the blood samples were taken from newborn babies' heels and were applied to five marked circles on newborn screening forms (made of S&S 903 filter paper; Schleicher and Schuell Inc.), allowed to dry, and then sent to the North Carolina State Health Laboratory by mail or courier service. The perinatal cards are not covered, so each card abuts directly against the next card in the file.

For this study, residual perinatal cards were retrieved from the archives and shipped from the State Health Laboratory wrapped in an individual plastic protective cover. A pair of scissors was cleaned by making at least 20 swipes in 10% bleach, then in deionized water, and finally in 70% ethanol. The residual ethanol was eliminated by blotting on a clean tissue. An entire 1-cm-diameter dried blood spot was cut from the perinatal card and placed in a labeled 15-ml conical tube. A blank spot cut from a clean piece of Schleicher and Schuell filter paper was treated in a similar manner, as a control for DNA contamination.

DNA Extraction

DNA was extracted from dried blood spots using the QIAamp DNA micro kit (Qiagen, Stanford, CA). This kit is designed to purify genomic DNA from small samples in four steps: sample lysis, binding of lysate to the silica-gel membrane of the QIAamp MinElute column, washing the membrane, and eluting the DNA. We modified the manufacturer's recommended method by using the whole dried blood spot (~1 cm diameter) instead of a smaller punch (~3 mm in diameter) from the blood spot. We also added a step in which additional lysate from the dried blood spot was collected using a forensic spin filter that uses centrifugal force to increase volume recovered from solid samples.

First, the perinatal card spot was submerged in 360 μl of Buffer ATL and 40 μl of proteinase K from the QIAamp DNA micro kit (Qiagen). The conical tubes were vortexed for 10 seconds and then incubated at 55°C for 60 minutes, tilting the tubes to maximize contact between the perinatal card and the buffer, and vortexed for 10 seconds approximately every 8 to 10 minutes. The perinatal card circle was retrieved using a pipette tip and then transferred into a forensic spin basket tube (part no. 2566; Alltech, Deerfield, IL) and spun in a microfuge at 6000 $\times$ *g* for 5 minutes at room temperature. After this procedure, the perinatal card circle was generally devoid of color. If it was not completely white, the circle was centrifuged again over the same forensic column to further capture any remaining lysate. The recovered lysate from this forensic filter tube was combined with the rest of the lysate from the original conical tube, mixed with 400 μl of buffer AL from the Qiagen kit, and incubated at 70°C with intermittent vortexing according to the manufacturer's instructions. The lysate was then purified over a Qiagen spin column according to the manufacturer's directions. Note that loading of the lysate onto the column required two spins of ~400 μl each due to the doubled volume of lysate (800 μl). The column was washed according to the manufacturer's instructions. DNA was eluted into 100 μl of AE buffer, and 10 μl was used in each amplification reaction except as noted.

Amplification

The purified DNA was tested in a multiplex, real-time PCR assay to detect a highly conserved segment of the HCMV polymerase (*POL*) gene and a control human gene (*APOB*) using primers and TaqMan probes described by Sanchez and Storch,[9] except we labeled the 5′ end of the *APOB* probe with TET (tetrachlorofluorescein) and the 3′ ends of both HCMV and *APOB* probes with the quencher dye TAMRA (carboxytetramethylrhodamine). The 5′ end of the HCMV probe was labeled with FAM (6-carboxyfluorescein). Primers and labeled TaqMan probes were purchased from TIB Molbiol LLC, Adelphia, NJ. Purified HCMV DNA was prepared by culturing early-passage Towne strain HCMV and purifying extracellular viral particles on a CsCl gradient. After extraction, the purified HCMV DNA was quantified against serial dilutions of a commercially available HCMV DNA standard (CMV AD169 quantitated viral DNA; Advanced Biotechnologies, Inc.). Five serial 10-fold dilutions of the Towne strain DNA from 80,000 to 8 copies of HCMV per PCR served as the standard for Q-PCR measurement of HCMV.

PCR was performed in a 50-μl volume containing TaqMan 2$\times$ Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 250 nmol each of the four primers, and 37.5 nmol each of the two labeled probes, 10 μl of template DNA, and nuclease-free water. Reactions were performed on a Prism 7900 sequence detection system (ABI) using universal cycling parameters as follows: after an initial hold at 50°C for 2 minutes, denaturation was at 95°C for 10 minutes followed by 45 cycles of denaturation at 95°C for 15 seconds and extension at 60°C for 1 minute. To check for amplicon contamination, multiple samples containing no template were included in every run.

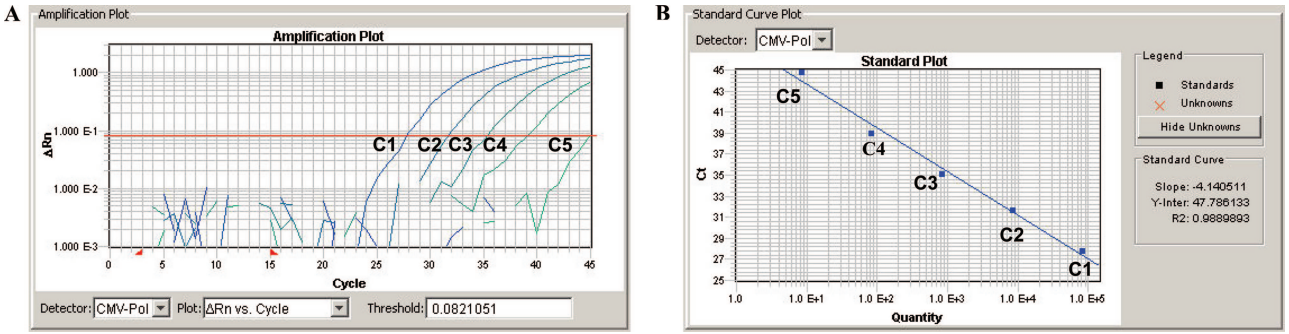

Figure 1. Real-time PCR of CMV DNA extracted and amplified from Guthrie cards reveals that the assay is sensitive and linear. **A:** Amplification plot shows change in fluorescence on the *y* axis (ΔRn) and PCR cycle number on the *x* axis. Standards contain varying levels of CMV DNA as follows: C1, 80,000 copies/5 μl blood; C2, 8000 copies/5 μl blood; C3, 800 copies/5 μl blood; C4, 80 copies/5 μl blood; C5, 8 copies/5 μl blood. **B:** Standard curve shows cycle threshold (Ct) on the *y* axis and template DNA level on the *x* axis. Correlation coefficient (R^2) = 0.989. Linearity drops off with the highest dilution (C5).

Results

The accuracy of the perinatal card extraction and amplification method for detecting HCMV DNA was evaluated by creating mock samples. Two blood samples drawn on 2 different days from an AIDS patient known to have active HCMV infection were chosen because of their high HCMV viral load (298,687 and 392,094 copies/ml of fresh blood). A third blood sample having no detectable HCMV DNA to a sensitivity of 400 copies per ml of fresh whole blood was used as a negative control. A known volume (50 μl) of fresh whole blood from each of the three patients was applied to filter paper in triplicate and then dried for at least 48 hours. DNA was extracted from the blood spots and PCR amplified. The percentage recovery of HCMV DNA from the filter paper based on the known copy number applied to the filter paper was 81% for both samples from the AIDS patient, whereas the negative control had no detectable HCMV DNA (data not shown). Control *APOB* DNA was amplified in all mock samples.

Assay Linearity and Sensitivity

Assay linearity on dried blood from perinatal cards was evaluated by preparing serial dilutions of HCMV DNA (Towne strain) into HCMV-negative whole blood at concentrations of 80,000 copies/5 μl blood (C1), 8000 copies/5 μl blood (C2), 800 copies/5 μl blood (C3), 80 copies/5 μl blood (C4), and 8 copies/5 μl blood (C5). A known volume (50 μl) of each dilution was applied to filter paper in triplicate spots. The DNA from each spot was extracted from the filter paper and 10 μl of the 100-μl elute was used in each PCR assay, equivalent to testing 5 μl of whole blood. Reproducibility was assessed by extracting and amplifying duplicates on 1 day and the triplicate spot on a different day. The correlation coefficient (R^2 value) for each of the three serial dilutions was 0.997, 0.989, and 0.996 (Figure 1). The mean threshold cycle (Ct) and SD of the reproducibility data for the Towne strain virus serial dilutions at each serial dilution was 27.4 ± 0.7 (C1), 31.5 ± 0.5 (C2), 35.3 ± 0.2 (C3), 39.6 ± 0.9 (C4), and 44.8 ± 0.1 (C5). The sensitivity of the PCR assay is eight copies of HCMV DNA per PCR reaction, based on the highest dilution of purified HCMV DNA that consistently yielded measurable results. This translates to a sensitivity of 1600 copies/ml of whole blood, or 80 copies of HCMV DNA in a 1.0 × 1.0-cm dried blood spot containing ~50 μl of whole blood. In conclusion, the extraction and amplification of HCMV DNA from perinatal cards is sensitive and linear over five-log concentrations of viral load.

Clinical Validation in Perinatal Card Samples

The perinatal cards of seven patients with culture-proven congenital HCMV infection were used as positive controls, whereas control infants whose cards were filed in front of and behind the cards from the positive control patients served as negative controls. A card on which no blood was spotted served as a control for amplicon contamination, and it showed no amplifiable HCMV or *APOB* DNA. HCMV DNA was detected in all seven positive control patient blood spots collected at less than 48 hours of age (Table 2) but was not detected in any negative control perinatal cards. The human *APOB* gene was amplified in all 24 negative controls (including 12 negative controls associated with the earliest collection time point of <48 hours, see *APOB* values in Table 2).

The HCMV PCR assay, as applied to blood spots on perinatal cards, is not quantitative because the volume of blood in each spot is undoubtedly variable. Furthermore, *APOB* level is not an ideal normalizer because the white cell count of each infant may vary, and the efficiency of extraction from each blood spot may vary. Nevertheless, if we assume that the blood spots contained similar volumes of blood and extraction was similarly efficient across all cases, then it is clear that the HCMV viral load varied considerably among the seven infants with congenital HCMV infection (Table 2). Higher cycle thresholds (suggesting lower viral loads) were evident in patients 3, 5, and 7. The lowest level was evident in patient 7 in whom the cycle threshold was reached very close to the end of the amplification cycles. The highest viral levels were seen in patients 1, 4, and 6. Remarkably, even the negative control card(s) directly abutting these patient's highly viremic cards contained no detectable HCMV DNA.

Table 2. Replicate Real-Time Amplification of the HCMV Polymerase Gene and the Human *APOB* Gene in Perinatal Cards Obtained at Less than 48 Hours of Age

Patient number*	HCMV *POL* cycle threshold†	*APOB* control cycle threshold†
Positive controls		
1	33, 34	34, 34
2	40, 40	32, 31
3	42, 43	36, 36
4	38, 37	31, 32
5	42, 38	32, 32
6	36, 36	36, 36
7	45, 43	31, 32
1 to 7	Mean: 39 ± 4 Median: 39 Range: 33 to 45	Mean: 33 ± 2 Median: 32 Range: 31 to 36
Negative controls		
8 to 19	Undetectable	Mean: 32 ± 2 Median: 31 Range: 31 to 36

*Patient number is random; perinatal cards were obtained anonymously from the State Health Laboratory and thus experimental laboratory results could not be correlated with clinical status.
†Replicates are shown for positive control patients.

Using 10 μl of starting template DNA was sufficient to detect all positive control patients. To determine whether even more starting template DNA was better for detecting samples with low viral load, all PCR assays were repeated using 20 μl of starting template. Although all seven positive control patients still had measurable HCMV DNA, control DNA amplification of the human *APOB* gene was not as robust, possibly because of PCR inhibitors associated with too much template DNA. To maximize the HCMV detection rate, it is recommended that the PCR assay be run in duplicate using 10 μl of template in each well.

In some infants, multiple blood cards were available for testing. In general, the viral loads were lower for the cards collected at less than 48 hours than at later time points in the same infant, with the caveat that there was no control for the amount of blood on each card or the efficiency of DNA recovered from each card (data not shown). All of the negative control cards filed in front of and behind these patient cards had undetectable HCMV DNA and amplifiable *APOB* DNA, with minimal *APOB* variation (data not shown).

Discussion

This is the first study to show that real-time amplification of HCMV DNA in perinatal cards is reliable for detecting congenital HCMV infection in neonates. Several groups of investigators have used conventional PCR to amplify HCMV DNA extracted from blood spots on perinatal cards.[4–7,10–14] The most recent studies[7,14] used the method of Barbi and colleagues,[4] which involves heat extraction followed by a nested PCR reaction amplifying the HCMV glycoprotein B (gB) gene and detection by agarose gel electrophoresis. This method was 100% sensitive and 99% specific compared with viral culture in 509 babies with congenital HCMV infection defined by culture.

In this study, HCMV DNA was detected in all seven cases of culture-proven congenital HCMV infection, whereas all negative control cases lacked detectable HCMV DNA. The number of patients tested was limited by the length of time the perinatal cards were stored in North Carolina (2 years after birth). The efficacy of DNA extraction and amplification in each sample was proven by successful amplification of a human *APOB* gene segment. We modified the manufacturer's recommended extraction method by using the whole dried blood spot (~1 cm in diameter) instead of a smaller punch (~3 mm in diameter) from the blood spot. The yield of sample lysate was increased by using a forensic spin basket filter to capture additional volume from the perinatal card. A major advantage of real-time PCR over the nested PCR method used by other investigators is the minimal risk of amplicon contamination and the less labor intensive, and therefore less costly, protocol. It should be noted that the sensitivity in our assay (1600 copies/ml) is not as low as that described in the nested PCR method (400 copies/ml).[15] Nevertheless, a real-time PCR method has analytical and clinical benefits, and it may be applicable to other laboratory assays targeting low-level DNA in blood card samples.

Although the assay is semiquantitative, it is probably not clinically relevant to report results of perinatal card testing in a quantitative manner. We suggest that viral PCR results be reported qualitatively as either "HCMV DNA was present in DNA extracted from the perinatal dried blood spot, consistent with a diagnosis of congenital HCMV infection" or "HCMV DNA was not detected in DNA extracted from the perinatal dried blood spot, suggesting no active HCMV infection at the time of neonatal blood collection." In the latter case, a diagnosis of congenital HCMV infection is not ruled out because infection occurring early in gestation may have resolved by the perinatal period. Also, a congenital infection (defined as acquired *in utero* or up to 3 weeks after birth) may be acquired after birth (for example, from exposure in the newborn nursery).

The frequency at which asymptomatic newborns have occult HCMV infection deserves study. Further studies

are also warranted on perinatal cards of patients with sensorineural hearing loss or other neurological deficits to determine the percentage attributable to congenital HCMV infection. Barbi and colleagues[5] used the nested PCR method to test Guthrie cards in children with sensorineural hearing loss, and 20 to 30% of cases were attributable to congenital HCMV infection. Congenital HCMV infection also causes other neurological sequelae such as microcephaly, seizures, mental retardation, paresis, and paralysis. Zucca and colleagues[7] recently retrospectively diagnosed congenital HCMV infection in 4 of 10 patients with malformations of cortical development by detecting HCMV DNA in dried blood spots. Our new assay provides a useful tool for similar retrospective clinical studies.

Testing perinatal cards is not recommended for diagnosis of active, symptomatic HCMV infection in a neonate. Instead, viral culture or PCR of fresh blood or body fluids/tissues are the diagnostic methods of choice. Perinatal card testing is indicated when congenital HCMV infection is in the differential diagnosis of a patient who has an appropriate clinical finding (eg, hearing loss or other neurological sequelae) that is recognized later in childhood when the active infection has resolved and current testing is no longer informative. We recommend testing all of the available blood cards that were collected during the first 3 weeks after birth, because it is possible that viral load increases throughout time as maternal antibodies wane in the infant. Although retrospective diagnosis of congenital HCMV infection is too late to influence antiviral treatment decisions, diagnosis of an infectious cause of a neurological disorder is reassuring to parents in that a genetic cause (although not completely excluded) is less likely. Therefore, subsequent pregnancies of the parents and patients are not expected to be affected. However, because infectious and genetic causes of hearing loss (such as connexin gene mutations or a genetic syndrome) could potentially co-exist, genetic causes of hearing loss or other neurological sequelae should still be pursued if clinically indicated. Finally, retrospective studies will provide valuable epidemiological information to further define the spectrum of neurological deficits attributable to HCMV infection and the percentage attributable to HCMV infection. Although perinatal card testing is considered a retrospective diagnostic test, Binda and colleagues[15] have recently proposed dried blood spot testing for congenital HCMV infection for the purpose of newborn screening. This application may allow timely diagnosis and treatment, even in the absence of concurrent signs or symptoms, and may improve long-term outcome.

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